MUTAGENESIS AND ADAPTED STRAINS TO THE GROWTH IN LIQUID OR SOLID SUBSTRATES

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Summary

Mutation by UV light resulted in the isolation of A. niger strains geared towards high pectinase production in either SSF or SFM but never in both. Water activity plays an essential role on the levels of activity of specific enzymes. Mutated strains showed growth and sporulation patterns distinguishable by morphometric techniques, when cultured on defined media.

Many enzymes which break down complex polymers are produced commercially or are being developed for such use. Biosynthesis of these enzymes is often controlled by carbon catabolic repression. Selection for mutants resistant to analogous of glucose has been used to isolate mutants which are deficient in carbon catabolic repression of these enzymes. For example, resistance to 2-deoxyglucose has been used to isolate deregulated mutants of *Trichoderma*, which overproduce cellulase [17] or as we shall see below, to select for mutants overproducing pectinases in either SSF or SmF.

Shankaranand *et al.* [2] suggested that microbial strains selected for SSF processes should be different to those selected for SmF processes. They found, for example, that in a collection of nearly 30 bacterial strains, the majority of them were good enzyme producers either in SSF or SmF but seldom in both. Antier *et al.* [1, 3] isolated UV mutants of wild strain *A. niger* C28B25 [4].

The selection phenotype was 2-deoxy-glucose (DG) resistance (DG^R) but in culture media with two different water activities (a_w = 0.99 or 0.96) such phenotypes were labelled to belong to classes: a) AW99 and b) AW96 (when a_w = 0.96 after adding 15% ethylene glycol). Mutants AW99 had an inverse correlation between their ability to produce pectinase by SSF on coffee pulp (a_w = 0.96) as shown by solid bars in figure 1 with respect to the production of pectinase by SmF shown as clear bars in the same figure. Apparently there was a trade off relation between each kind of those phenotypes. Strain WT in that figure corresponds to wild type (C28B25) isolated by Boccas *et al.* [4]. Therefore, strain AW99-iii had seven times less potency for SSF pectinase production and more than three times more potency for SmF as compared to WT (see figure 1).

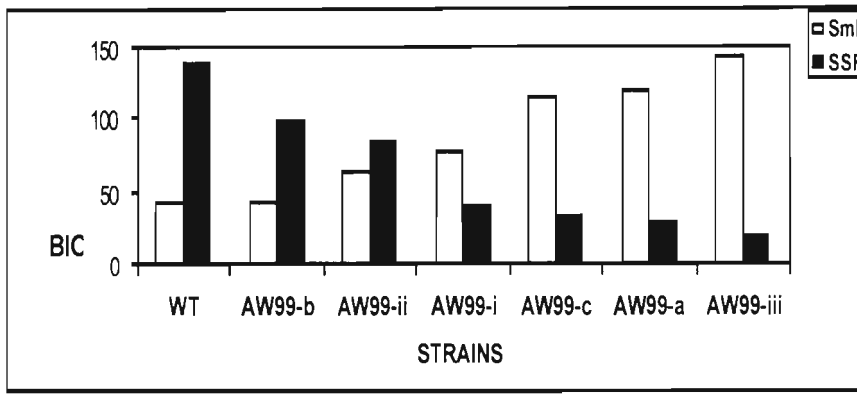

Figure 1. Comparison of pectinase activities of DG^R mutants of *A. niger* C28B25 isolated at high water activity (a_w = 0.99) and cultured in shake flasks (SmF) or coffee pulp packed bed columns (SSF) according to Antier *et al.* [1]. U PEC = arbitrary enzyme viscometry units expressed by g of solid substrate (SSF) or dry biomass (SmF).

In Figure 2 the same kind of results are shown for AW96 mutants. Here the inverse correlation of pectinase production by SSF and SmF is not as evident as in Fig. 1. Strain WT is the same wild type shown in the previous figure. All AW96 mutants had equal or higher potency in SmF than the wild type but some of them (strains AW96-1 and 3 in Fig. 2) increased their potency in SSF by nearly 40% over WT.

Antier *et al.* [3] showed that DG^R phenotype was independent from AW96 and AW99 phenotypes. Since a DG^R reverting strain (AW96-3 became DG^S) was found to retain high pectinase productivity in low water activity but became highly sensitive to DG as the wild type [3].

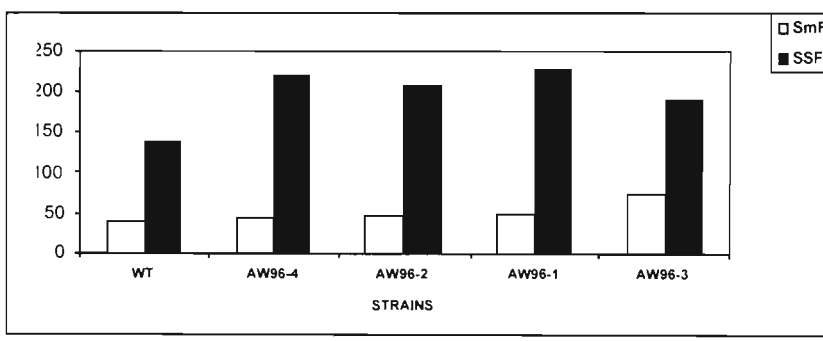

Figure 2. Comparison of pectinase activities of DG^R mutants of *A. niger* C28B25 (WT) isolated at low water activity (a_w = 0.96) and cultured in shake flasks (SmF) and coffee pulp packed bed columns (SSF) according to Antier *et al.* [1]. U PEC are arbitrary enzyme units by viscometry expressed by g of solid substrate (SSF) or dry biomass (SmF).

Recent work in our laboratory (Romero *et al.*, 1996 and 1997, unpublished data) has shown that strains AW96 and AW99 are derepressed for the production of pectinase, invertase and amylase, although they were selected on the basis of pectinase over production [1, 3].

Such observations support the existence of general regulatory mechanisms involved in the adaptation of moulds to liquid or solid environments and controlling the yield and quality of enzymes best suited for each kind of culture medium. As a consequence, the selection of strains for the production of enzymes by SSF technique requires the use of specific protocols including the survival to metabolic stress factors, namely, the presence of antimetabolites such as DG [1] or dinitro phenol [3] and also low levels of water activity.

Concerning the importance of water activity for the production of enzyme by SSF technique, Narahara *et al.* [13] proposed the use of mixtures of steamed rice particles with lignocellulosic particles in order to increase the moisture content of koji SSF fermentation for the production of amylases. Oriol *et al.* [11] used mixtures of steam cooked cassava granules with sieved fibres of sugar cane bagasse. This was based on the fact that bagasse can absorb four-fold its dry weight in water whereas cassava granules can only absorb one-fold its dry weight in water. The overall moisture content of those mixtures was a weighed average of the corresponding fractions of each material. Thus, initial moistures could be adjusted in the range from 42% up to 70%. A summary of their results is shown in Table 3. Similar results were obtained using different levels of glucose absorbed in bagasse, from 10 to 300 g/L [11].

The value of the specific growth rate was also an increasing function of a_w. In this system there was an optimum value for enzyme productivity (not shown in Table 2) because bagasse fibres did not contain starch nor were inoculated with mould spores, they were only used as a water reservoir. This experiment showed the presence of inter-particle mass transfer of moisture and suggested the use of mixed materials in order to correct for low moisture content in amylaceous substrates.

Table 3. Effect of initial water activity (a_w.) on the maximal amount of biomass (X_{max}) and substrate conversion of *Aspergillus niger* No. 10 [11].

Initial a_w.	X_{max} g/g Initial Dry Weight	SC g/g Initial Dry Weight
0.933	0.123	0.233
0.940	0.172	0.344
0.958	0.251	0.417
0.957	0.324	0.477
0.980	0.339	0.558

Pandey [12] in his review of SSF processes comments "the types of micro-organisms that can grow in SSF systems are determined by the water activity factor a_w. ... defined as the relative humidity of the gaseous atmosphere in equilibrium with the substrate"..."The micro-organisms, which can grow and are capable of carrying out their metabolic activities at lower

a_w values are suitable for SSF processes". Sarrete *et al.* [14] showed that addition of glycerol to a tempeh SSF fermentation by *Rhizopus oligosporus* changed the yield of various polysaccharidases having different optimal conditions, for example, polygalacturonases and xylanases were maximal for a_w values between 1.00 and 0.99 but endocellulase production was maximal when a_w = 0.98. Acuña-Argüelles *et al.* [15] used ethylene glycol as water depressor of SSF cultures of *A. niger* grown on bagasse as support. They found that decreasing values of a_w from 0.98 to 0.90 resulted in decreasing activities of exopectinase, as shown in Table 4.

Those results are in agreement with earlier observations [16] showing that the production of polygalacturonase and cellulase by *Geotrichum candidum* was very sensitive to a_w reduction, from 1.00 to 0.98, by the addition of KCl, mannitol or polyethylene glycol. Also, Grajek and Gervais [10] found that the production of polygalacturonase, D-xylanase and β-galactosidase by SSF cultures of *Tichoderma viridae* TS was influenced by water activity.

Table 4. Effect of water activity on the level of exopectinase produced by *A. niger* CH4, [15].

Water activity	Exopectinase activity (end point mg of reducing compounds per ml)
0.98	20
0.97	18
0.94	15
0.90	5

Minjares-Carranco *et al.*, [8] carried out physiological comparisons between pectinase-producing mutants of *A. niger* adapted either to SSF or SmF through morphometric analyses. Parent and mutant strains were grown on a specific medium and morphological measurements were performed with a digital image analyser. For the characterisation of each strain, two indexes were used Ib (growth rate of a colony on a given medium relative to maximun growth rate on medium DEX) and Ia (sporulated area over total area of colony after 72 hrs of growth). Each strain was then represented by a plot on the Ia versus Ib plane. Figure 3 shows the « mapping » of the wild type strain and the mutant strains. They map in three distinct regions.

Although the mutants described above were selected for their resistance to 2-deoxyglucose, the resulting pectinase-producing strains from both classes (AW99 and AW96) show distinct physiological and phenotypic patterns. The deoxyglucose resistance phenotype may not be directly involved with the complex patterns of physiological derepression and enzyme production.

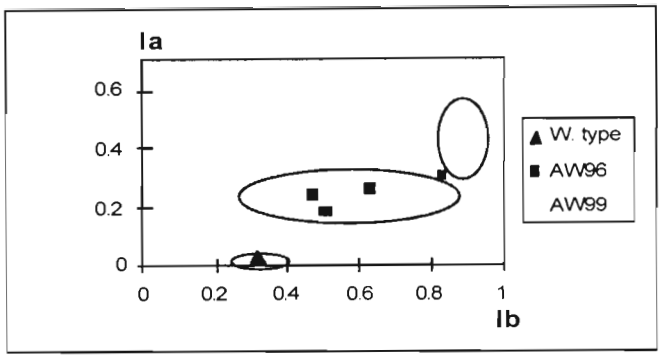

Figure 3.- Ia and Ib values obtained for wild type (triangles) and mutant strains of series AW96 (square) and AW99 (circle). Each plot represents the average of triplicate measurements on a given strain.

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